Insecticidal activity of daphnane diterpenes from *Lasiosiphon kraussianus* (Meisn) (Thymelaeaceae) roots

Adil EA Bala,† Robert Delorme,* Albert Kollmann, Lucien Kerhoas, Jacques Einhorn, Paul-Henri Ducrot and Danielle Augé

Unité de Phytopharmacie et Médiateurs Chimiques, INRA, Route de St Cyr, 78026 Versailles Cedex, France

Abstract: A methanol extract of roots of *Lasiosiphon kraussianus* (Meisn) (Thymelaeaceae) showed potent insecticidal activity against *Aphis gossypii* (Glov) and *Drosophila melanogaster* (Meig). Bioassay-driven fractionation of this extract led to the isolation and characterisation of two known daphnane diterpenoids: Excoecaria toxin (1) and wikstrotoxin D (2). The two natural products were inferior to methomyl in activity against *A gossypii* and *Myzus persicae* (Sulz) in contact assays but were superior in ingestion assays against *D melanogaster*. This is the first report on insecticidal activities of compounds 1 and 2. AChE was insensitive to the two natural products.

Keywords: *Lasiosiphon kraussianus*; daphnane; insecticidal activity

1 INTRODUCTION

Development of insecticide resistance has become a well-known phenomenon among most organochlorine, organophosphorus, carbamate, formamidines and pyrethroid insecticides. This is attributed to the extensive and indiscriminate use of these chemicals.[1] In addition, many pesticides have been restricted in their use or prohibited, due to concerns over environmental and food safety.[2] Natural products from plants still have enormous potential to inspire and influence modern agrochemicals research. They are considered to be less likely to harm ecosystems than conventional insecticides, and are often selective in their toxicity. It is estimated that, to date, only about 10% of plant species in the world have been examined chemically.[3] There is therefore, a challenging task to discover new pesticides classes and/or new modes of action from natural plant sources.

Plants produce, through unique pathways, secondary metabolites to protect themselves from the attack of various herbivores.[4] These compounds represent an enormous diversity of biologically active compounds. Screening of plant preparations, followed by a bioassay-guided fractionation, leading to isolation and identification of pure active plant constituents, is considered to be one of the most successful methodologies for the discovery of new products. In this case, it is much more likely that plant natural products will be used as a lead for synthesis rather than as products *per se*.[3]

The present screening programme was carried out with wild plants from Sudan. One of the tested materials was the methanol extract of the roots of *Lasiosiphon kraussianus* (Meisn) (Thymelaeaceae), traditionally known to be a toxic plant. The Thymelaeaceae is a moderate sized family of dicotyledons consisting of nearly 1200 species distributed in 67 genera. Members of this family are found throughout the tropical and temperate parts of the world, and are absent only from the coldest climate.[5] The genus *Lasiosiphon* itself is not clearly delineated; some authors include species of the genus *Lasiosiphon* as a part of *Gnidia*, while others have merged the genera *Lasiosiphon* and *Arthrosolen* into *Gnidia*.[6] The woody herb *L kraussianus* is distributed in Africa from Guinea to north Nigeria eastward to the central and southern states of Sudan and south through east Africa to Mozambique, Zambia and Botswana.[7] A detailed description of this genus had been reported.[8] In fact, some pharmacological investigations have been carried out on the roots of this plant, and potent activities against leukemia strains[6,9,10] and other diseases[11] were reported, but, so far, no insecticidal activity has been reported. The present work describes the purification, identification and insecticidal activity of two constituents isolated from this root extract.

* Correspondence to: R Delorme, Unité de Phytopharmacie et Médiateurs Chimiques, INRA, Route de St Cyr, 78026 Versailles Cedex, France
†On leave from University of Gezira, PO Box 20, Wad Medani, Sudan
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2 MATERIALS AND METHODS

2.1 Plant collection and preparation

The vernacular name of *L kraussianus* in Darfour (western Sudan) is Komma or Mahjiria. The roots of this plant were collected in November 1994 at Jebel Marra (Wadi Mertagello, 1160 metres above sea level), and identified by K Uhlig and A A Adam, at the Jebel Marra Forest Circle, Golol, GTZ Project, Sudan. A voucher specimen is available in their herbarium. The roots were first cut in small pieces, dried under shade for two weeks and then milled to a powder form.

2.2 Extraction and fractionation

2.2.1 Extraction

All solvents and chemicals used were of analytical grade. One hundred grams of powdered roots were placed in a conical flask (2 litres). Methanol (1.2 litre) was added, the flask shaken for 2 h and then kept overnight in a refrigerator. After filtration under vacuum through Hyflosupercel (Prolabo, France), the dried de-fatted debris was percolated with methanol (600 ml). The filtrate and the percolate were combined and concentrated using a rotary evaporator (water-bath temperature, 40 °C). The residue was finally dissolved in methanol (25 ml) from which 5 ml were used for the crude bioassay. The rest (20 ml) was used for the fractionation step.

2.2.2 Fractionation

The 20 ml of the methanol extract was mixed with water (40 ml) and partitioned with dichloromethane (2×60 ml) in a separatory funnel. Both fractions were concentrated, taken into methanol and subjected to a bioassay. The non-polar fraction, where the activity was found, was then chromatographed on a silica gel (Kieselgel) column, (40 cm long $\times$ 2.5 cm ID), packed with 80 g of 0.063–0.125 mm (120–230 mesh ASTM) in a slurry form in dichloromethane. For elution, the fraction was subjected to gradient chromatography, starting from dichloromethane to dichloromethane + ethyl acetate + methanol (60 + 20 + 20, by volume) at a constant flow rate of 3 ml min^{-1}, using HPLC (Waters 600, multisolvent delivery system) equiped with a fraction collector (Sieve type 192-N). Sixty tubes (18×180 mm) of 17 ml each were collected. All tubes were subjected to a TLC single-spot analysis (Polygram sil G/ UV 254, layer thickness 0.25 mm (Macherey-Nagel) and developed with hexane + ethyl acetate (30 + 70, by volume), followed by visualization under UV light at 254 nm and, then collected in 11 fractions.

The active fraction was subjected to gel exclusion over Sephadex LH-20. The conditions were: column: 90 cm $\times$ 2.5 cm; Sephadex LH-20 (Pharmacia) particle size 25–100 μm; slurry-packed in methanol; mobile phase: methanol; flow rate: 2 ml min^{-1}. The pump used was Gilson 301; 2138 Uvicord S detector (LKB), wavelength 254 nm; 2-channel recorder LKB. Fraction collector used was Foxy, Isco. Fifty-six tubes (13×100 mm) of 7 ml each were collected. After TLC analysis, all tubes were collected in six sub-fractions. The active sub-fraction was then subjected to preparative reversed-phase HPLC (Waters 600, multisolvent delivery system). The isocratic conditions were: flow rate: 3.7 ml min^{-1}, mobile phase: methanol + water (74 + 26 by volume). Each peak was collected manually and the two purified active peaks were then subjected to identification and bioassay.

2.3 Spectroscopic analysis

2.3.1 Mass spectrometry (MS)

The MS and MS-MS spectra were obtained with a Nermag R 30-10 (Quad Service, Poissy, France) triple quadrupole instrument. Source conditions were set as follows: temperature, 130 °C; filament current, 50 μA; electron energy, 95 eV; reagent gas (NH_3 or ND_3) pressure was set at 10^{-4} Torr in the source housing. For MS-MS experiments, the collisional activated dissociation (CAD) spectra were obtained at 20 eV collision energy and with argon (7×10^{-2} Torr) as collision gas in the second quadrupole. Sample introduction was done by desorption chemical ionization (DCI) in both positive (PICI) and negative (NICI) mode.

2.3.2 [^{1}H]NMR

Both compounds were subjected to [^{1}H]NMR analysis (Varian Gemini, 300 MHz) in solution in deuterochloroform using residual chloroform as an internal reference (7.27 ppm).

2.4 Bioassay procedures

2.4.1 Rearing conditions of insects and mites

The susceptible strains of all insects, *Aphis gossypii* (Glov), *Myzus persicae* (Sulz), *Drosophila melanogaster* (Meig), *Spodoptera littoralis* (Boisd), *Sitophilus granarius* L and the spider mite *Tetranychus urticae* (Koch), were used for the bioassay. They were reared under controlled conditions [20 (± 1) °C, 60 (± 10)% RH and 8/16 h L/D period]. *A gossypii* was reared on cucumber seedlings, *M persicae* on broad bean, *Drosophila* and *S littoralis* on semi-synthetic diets, *S granarius* on wheat and *T urticae* on bean seedlings.

2.4.2 Spraying bioassay against Aphis gossypii and Myzus persicae

This test was carried out in small boxes (2.7 cm diameter). These boxes were filled with agar-agar (semi-solid gel). Cucumber leaf discs (broad bean in case of *M persicae*) were placed on the agar. Two to three hours before the treatment, 15 *A gossypii* adults (apterae) or 20 first-instar larvae in the case of *M persicae* were placed on each box for adaptation. Two boxes were used for each dose. Deionised water + acetone (80 + 20, by volume) was used for the preparation of a logarithmic series of five dilutions for each product. For each concentration, 0.5 ml was directly sprayed on to the two boxes using a micro-

sprayer (acetone + water in the case of the control). The boxes were then placed under the above-mentioned controlled conditions.

The dead aphids were counted 24 h (*A gossypii*) or 48 h (*M persicae*) after the application. Insects were judged to be dead if they were on their sides or backs, had their legs folded over the ventral surface, showed signs of desiccation, and/or did not respond to prodding with a paint brush.[12] Percentage kill (in all tests below also) was corrected for control mortality by Abbott's formula and the LC_{50} values were calculated using probit analysis,[13] with a special microcomputer programme.[14]

2.4.3 Activity against Drosophila melanogaster

2.4.3.1 Incorporation into diet. The overall toxicity test was carried out in small bottles (7 cm high, 4.2 cm diameter). Thirty-five grams of a semi-synthetic diet [bran (2.2 litre), sugar (260 ml), yeast (130 ml), vinegar (70 ml), nipagine (20 ml), and water (580 ml)] was placed and pressed into each bottle. Each treatment was replicated twice. In each bottle, 1 ml of each concentration was incorporated into the diet (acetone + water in case of control). Two to three hours after the treatment (after acetone evaporation), 15 adults, zero to three days old (10 females and five males), were placed in each bottle. The bottles were then plugged with cotton and returned to the rearing conditions. The adults were removed from the bottles when some of the new nymphs in the control developed to pupae (about 13 days). The number of adults were counted every two days for two weeks (seven readings). Emergence percentage was corrected for the control and, LC_{50} values were calculated using the above-mentioned methods.

2.4.3.2 Topical application. Petri dishes (6 cm diameter) were first washed with diethyl ether. Small cubes of gel agar media (10 g $litre^{-1}$ agar + small amount of honey) were placed in each Petri dish. The insects were placed in a deep-freezer for about 2.5 min. For each concentration, 0.3 µl (methanol in case of control) was applied on the thoracic tergites using an automatic microapplicator (Burkhard Manufacturing Co Ltd, UK). Ten insects were used in each treatment (including the control), and these were replicated three times. After treatment, test insects were returned to their rearing conditions. Mortality was recorded after 24 h.

2.4.3.3 Contact application. Petri dishes (6 cm diameter) washed with diethyl ether were used. At each concentration (methanol in case of control), 0.5 ml was placed in each dish. After solvent evaporation, small cubes of the above-mentioned diet were placed in the dish and 30 insects were introduced. Each treatment was replicated three times. Mortality was recorded after 24 h.

2.4.4 Activity against Spodoptera littoralis

2.4.4.1 Topical application. Third-instar larvae (35 (± 10) mg) were used. At each concentration (methanol in case of control), 0.5 µl was topically applied on the dorsum of each larva. Each treated larva was placed in a separate box (2.7 cm diameter), provided with untreated food and then returned to its rearing conditions. Ten larvae were used for each treatment, (including the control) and these were replicated twice. Mortality was recorded after 24 h. The effects on growth and development were monitored by rearing the larvae and recording the time taken for adults to emerge and the numbers emerging.

2.4.4.2 Ingestion toxicity. The synthetic diet used consisted of water (3.2 litre), agar-agar (80 g), maize flour (300 g), wheat germ (126 g), yeast (134 g), cabbage leaf powder (200 g), ascorbic acid (18 g), benzoic acid (7.2 g), nipagine (7.2 g), salt mixture (10 g), vitamins (30 g), fumidil (5 g), formaldehyde (1.5 ml), linseed oil (8 ml). Third-instar larvae were starved for 24 h prior to exposure to the diet. To each small cube (200 mg) of semi-synthetic diet, 250 µl of each concentration (methanol in case of control) was added. After solvent evaporation, one treated cube was placed individually into each small Petri dish (2.7 cm diameter). Larvae were allowed to feed on the treated diet for 24 h (10 larvae for each concentration) and were then presented with untreated diet. Mortality was determined 48 h after treatment. The insects were observed up to the emergence of new adults to record the effect on growth and development.

2.4.5 Activity against Sitophilus granarius: topical application

In this test, 15-day-old adults were used. An aliquot (0.5 µl) of each concentration (methanol for control), was topically applied on the dorsum of each insect using the microapplicator. For each treatment, 25 treated insects were placed in small bottles (7 cm high, 4.2 cm diameter) with a small amount of wheat and the bottles were closed with fine nylon mesh. Each treatment (including the control) was replicated twice. Mortality was recorded after 24 h. For effects on reproduction, adults were removed from the bottles after two weeks and the number of newly emerged insects was recorded for a period of eight weeks. The total number of newly maintained insects was compared to that of the control as mentioned above.

2.4.6 Activity against Tetranychus urticae

2.4.6.1 Against adults. The method adopted in this test was to some extent similar to that for aphids: small boxes (2.7 cm high, 4.5 cm diameter) were filled with agar-agar (semi-solid gel) and bean leaf discs were placed on the agar. Thirty adults were placed on each box 3 h before treatment, for adaptation. Two boxes were used for each concentration. At each concentration (methanol in case of control), 0.5 ml was directly sprayed into the two boxes. The treated mites were

Species	Application method	Application rate[a]	Corrected mortality[b] (%)
A gossypii	Spraying	0.08 mg plant per ml^{-1}	83
D melanogaster	Contact	70 mg plant cm^{-2}	93
	Topical	1.5 mg plant per insect	0
	Ingestion	1000 mg plant ml^{-1}	100
S granarius	Topical	2 mg plant per insect	0
S littoralis	Topical	2 mg plant per insect	0
	Ingestion	1 mg plant per insect	0
T urticae (Adults)	Spraying	0.08 mg plant ml^{-1}	0
T urticae (Eggs)	Spraying	0.08 mg plant per ml^{-1}	42

[a] The amount given pertains to the quantity of plant material extracted.
[b] According to Abbott's formula.

Table 1. Activity of methanol extract of the *Lasiosiphon kraussianus* root against some insects and one mite species

then placed in their controlled conditions. Mortality was recorded after 24 h.

2.4.6.2 Against eggs. Twenty adults were placed in the boxes mentioned above for 48 h. The adults were then removed and the eggs were counted. Two boxes were used for each concentration. At each concentration (methanol in case of control), 0.5 ml was directly sprayed to the two boxes. In all cases, the boxes were returned to the controlled conditions. The number of unhatched eggs and dead larvae was recorded for seven days.

2.5 Anticholinesterase activity

Acetylcholinesterase (AchE) activity of *A gossypii* was measured using acetylthiocholine iodide as substrate and determining the released thiol colorimetrically by its reaction with 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB).[15]

3 RESULTS AND DISCUSSION

3.1 Activity of the crude extract

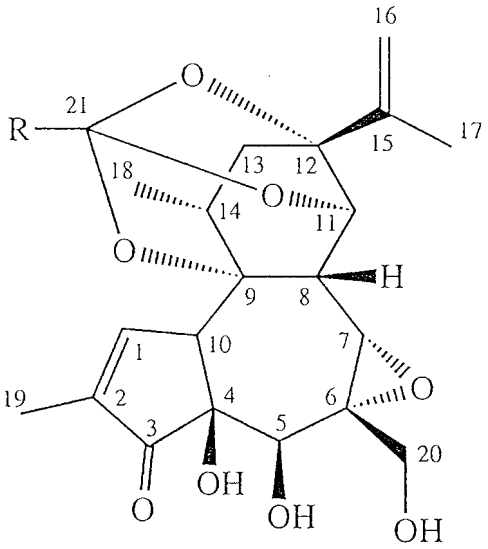
The bioactivity of the crude extract was evaluated against four insects using different routes of administrations, and one mite species. This crude extract exhibited a potent efficacy (Table 1). Against *A gossypii*, it caused 83.3% mortality after 24 h (80 µg plant ml^{-1}). Against *D melanogaster*, the crude extract scored 100% when incorporated into diet (1 g plant ml^{-1}), and 93% following tarsal contact application (4 g plant ml^{-1}) and 41.7% when applied against the eggs of *T urticae* (80 µg plant ml^{-1}). No activity was recorded against *S littoralis* and *S granarius*.

3.2 Purification and identification

The crude extract was partitioned and the activity was found in the non-polar fraction which was fractionated on silica gel. Bioassay-directed fractionation by HPLC was carried out using *A gossypii*. Activity was detected in two peaks which had retention times (R_t) of 28.84 min and 53.90 min. The structures of the compounds in each peak were identified and the activity of the compounds was determined against three insects: *A gossypii*, *M persicae* and *D melanogaster*.

The two active compounds were identified by NH_3-DCI-MS (and -MS-MS) and NMR spectroscopy as diterpenoids with a daphnane skeleton: excoecaria toxin **(1)**[6] $[\alpha]_D + 61.0°$ (C = 0.190, CH_2Cl_2) and wikstrotoxin D **(2)**[16] $[\alpha]_D + 17.3°$ (C = 0.643, CH_2Cl_2) (Fig 1). The molecular masses were assigned respectively as 528 and 532 according to positive and negative NH_3-DCI (ions at *m/z* 529 and 533, being the MH^+ species and *m/z* 528 and 532 corresponding to $M^{-\circ}$, respectively). The ND_3-DCI spectra in the positive mode, exhibiting ions at *m/z* 533 and 537, confirmed these assignments and indicated further the presence of three exchangeable (likely hydroxylic) hydrogens in both compounds.

In order to obtain structural information and more evidence of these assumptions, the CAD spectra (eg MS-MS experiments) of the MH^+ ions (in comparison with the corresponding M_DD^+ ions) were performed. The decomposition was observed to occur through two main pathways leading to: (i) the formation of an ion either *m/z* 151 (**1**) or 155 (**2**)



1 R = -(CH=CH)$_2$-(CH$_2$)$_4$-CH$_3$ (*E,E*)

2 R = n-C$_9$H$_{19}$

Figure 1. Chemical structures of excoecaria toxin (**1**) and wikstrotoxin D (**2**).

Insect species	*Product*	LC_{50} ($\mu g\,ml^{-1}$)	*95% Confidence limits* ($\mu g\,ml^{-1}$)	*Slope of regression line*
A gossypii (spraying)	Excoecaria toxin	19	12–28	1.2
	Wikstrotoxin D	17	11–26	1.2
	Methomyl	2.0	1.6–2.5	2.2
	Deltamethrin	0.057	0.046–0.070	2.1
M persicae (spraying)	Excoecaria toxin	90	59–140	1.8
	Wikstrotoxin D	53	47–60	3.9
	Methomyl	7.9	5.8–11	3.5
	Deltamethrin	0.032	0.024–0.042	3.4
D melanogaster (into diet)	Excoecaria toxin	19	13–27	1.1
	Wikstrotoxin D	23	14–36	0.76
	Methomyl	39	28–53	1.3
	Deltamethrin	1.4	0.74–2.8	1.7

Table 2. Insecticidal activity of excoecaria toxin and wikstrotoxin D

which was interpreted as related to the lateral aliphatic chain (unsaturated or not) corresponding to a RCO^+ ion, and (ii) the elimination of a RCO_2H neutral giving rise to an ion at *m/z* 361 in both compounds. Further evolution of the latter includes losses of H_2O (up to 3 molecules), CO, CH_2O and C_3H_6 (or CH_2CO) in various combinations. These data were in agreement with the above proposed structures.

[^{1}H]NMR spectra were then recorded and found to yield data identical to those reported previously in the literature.[6,16]

3.3 Activity of excoecaria toxin and wikstrotoxin D against insects

The two purified natural products (excoecaria toxin and wikstrotoxin D) were subjected to a comparative evaluation (bioassay) with two well-known insecticides: deltamethrin (pyrethroid) and methomyl (carbamate). Both natural products showed dose-dependent relationships as insecticides against *A gossypii, M persicae* and *D melanogaster* but no activity against *S littoralis or S granarius*. However, the purified products were not tested for their activity against *T urticae* (eggs). They were inferior to methomyl against *A gossypii* and *M persicae* in contact assays but superior in ingestion assays against *D melanogaster*. Deltamethrin proved to be the most potent (Table 2). These results indicate that the daphnane diterpenoids have some selectivity in their activity.

3.4 Anticholinesterase activity

Understanding how pesticides achieve their toxicity requires knowledge about their sites of action. The AChE activites when using excoecaria toxin and wikstrotoxin D were measured. AChE was found to be insensitive to either of these two products (only 6% and 4% at 5×10^{-5}M for excoecaria toxin and wikstrotoxin D, respectively) and may be to all daphnane diterpenoides, compared to methomyl (83% at 10^{-5}M). It can be said that the site of action of these new potential insecticides could be in another system.

4 CONCLUSION

The results of these experiments clearly demonstrate, for the first time, the potent insecticidal activity of the two natural daphnane diterpenoids. Excoecaria toxin was previously identified in *G kraussiana* (apparently identical to *L kraussianus*). However, it is the first time that wikstrotoxin D has been reported in *L kraussianus*. On the other hand, the methanol extract of this plant root at a dose of $2\,mg\,kg^{-1}$ *per os*, does not cause any toxic effect in mice, rats and rabbits[11] and, in addition, these two natural products have been reported as anti-tumour compounds.[16]

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